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Underestimation of Vancomycin and Teicoplanin MICs by Broth Microdilution Leads to Underdetection of Glycopeptide-Intermediate Isolates of *Staphylococcus aureus*▽†

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Broth microdilution was compared with tube macrodilution and a simplified population analysis agar method for evaluating vancomycin and teicoplanin MICs and detecting glycopeptide-intermediate isolates of *Staphylococcus aureus*. Modal vancomycin and teicoplanin MICs recorded by tube macrodilution and the agar plate assay, which both used inocula of 10^6 CFU, were significantly higher (2 μg/ml) against a panel of borderline glycopeptide-susceptible and glycopeptide-intermediate methicillin-resistant *S. aureus* (MRSA) bloodstream isolates compared to broth microdilution (1 μg/ml). Vancomycin and teicoplanin MIC distributions by tube macrodilution and agar testing were also markedly different from those evaluated by broth microdilution. The 20-fold-lower inoculum size used for broth microdilution compared to macrodilution and agar MIC assays explained in part, but not entirely, the systematic trend toward lower vancomycin and teicoplanin MICs by microdilution compared to other methods. Broth microdilution assay led to underdetection of the vancomycin-intermediate *S. aureus* (VISA) phenotype, yielding only three VISA isolates, for which vancomycin MICs were 4 μg/ml compared to 8 and 19 VISA isolates detected by macrodilution and agar testing, respectively. While macrodilution and agar testing detected 7 and 22 isolates with elevated teicoplanin MICs (8 μg/ml), respectively, broth microdilution failed to detect such isolates. Detection rates of isolates with elevated vancomycin and teicoplanin MICs by macrodilution and agar testing assays were higher at 48 h than at 24 h. In conclusion, the sensitivity of broth microdilution MIC testing is questionable for reliable detection and epidemiological surveys of glycopeptide-intermediate resistance in *S. aureus* isolates.

Since 1997, two major categories of vancomycin resistance in *Staphylococcus aureus* have been defined. The first category refers to vancomycin-resistant *S. aureus* (VRSA) clinical isolates with exogenously acquired, *vanA*-mediated high-level resistance (vancomycin MICs, ≥16 μg/ml) (7, 45); the second category includes vancomycin-intermediate *S. aureus* (VISA) isolates that developed low-level resistance (vancomycin MICs, ≥4 to <16 μg/ml) via complex, incompletely defined endogenous mechanisms (6, 10, 21, 51). Since VISA isolates are almost uniformly cross-resistant to teicoplanin (21, 30), they are frequently designated glycopeptide-intermediate *S. aureus* (GISA) (50). In contrast to vancomycin, widely different teicoplanin susceptibility breakpoints have been proposed by different national or international committees, varying from 2 (13) to 8 (10) μg/ml, which leads to a confusing situation.

Soon after their initial discovery in Japan (23), it was realized that a large proportion of VISA isolates, referred to as hVISA, show heterogeneous expression of vancomycin-intermediate resistance, including a minority population (perhaps as few as 10^{-6} cells) for which the vancomycin MIC is ≥4 μg/ml, while the majority of bacteria are still vancomycin susceptible (vancomycin MICs, ≤2 μg/ml) (10, 21, 22, 24, 51). No mechanistic model explaining heterogeneous expression of glycopeptide resistance has been provided. hVISA/hGISA are assumed to be precursors of VISA/GISA strains, with glycopeptides providing the selective pressure for conversion (2, 14, 22, 24, 33, 39, 44, 55). On the other hand, serial passages on antibiotic-free media frequently lead to gradual dilution and eventual elimination of the resistant subpopulation (2, 21, 24). These data potentially challenge the previously established distinction between hGISA and GISA (21, 29, 51).

Despite repeated efforts to create one, there is no standard molecular or phenotypic assay allowing reliable detection of GISA and hGISA clinical or laboratory isolates (5, 30). This situation can be explained by (i) the multifactorial molecular basis of hGISA/GISA phenotypes, which did not reveal any ubiquitous, single, specific molecular marker for their detection (24–27, 41), and (ii) the variable, phenotypic expression of low-level glycopeptide resistance, which is significantly influenced by several technical parameters, including the compositions of liquid or solid test media and varying time frames and inoculum sizes.

Standard CLSI-recommended broth microdilution and agar

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MIC-testing methods (9) were reported to have suboptimal sensitivity for detecting some hGISA isolates (21, 51) because they use relatively small inocula (5×10^4 CFU/well and 1×10^4 CFU/spot, respectively). Accordingly, specifically designed agar screening or population analysis profiles, as well as modified Etest methods, were developed for improved detection of hGISA and GISA by integrating requirements for larger bacterial inocula and longer incubation periods (5, 17, 24, 48, 51, 54, 58, 60). Nevertheless, standardization of these elaborated, labor-intensive susceptibility test methods is difficult (17, 48, 58, 60), and their relationships with standard glycopeptide MIC breakpoints are not well defined. Finally, the recent revisions of vancomycin MIC breakpoints by CLSI (10) and of both teicoplanin and vancomycin MIC breakpoints by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (13), which were based on glycopeptide susceptibility surveys of *S. aureus* clinical isolates (15, 51, 59), hamper analysis of hGISA/GISA prevalence data reported before 2006.

Despite the lack of standardized hGISA detection methods, a number of clinical reports have linked vancomycin therapeutic failure of methicillin-resistant *S. aureus* (MRSA) infections with the presence of VISA or hVISA isolates or with emergence of vancomycin-intermediate resistance during glycopeptide therapy (3, 8, 24, 25, 33, 36–38, 44, 51, 52). Even higher rates of vancomycin treatment failures were reported for bacteremic patients infected with MRSA isolates for which vancomycin MICs (2 μg/ml) were still in the susceptible range than for those with lower vancomycin MICs (<2 μg/ml) (3, 11, 18–20, 31, 32, 34, 35, 43, 46, 51). An emerging creep of vancomycin and teicoplanin MICs against MRSA in the last decade, which was suggested by large-scale epidemiological studies (19, 28, 47, 51, 56), has been challenged by more recent data (1, 42). Collectively, most of the discrepancies in the clinical and epidemiological results might have resulted from the lack of reliable, sensitive detection methods for hGISA and GISA.

During a retrospective surveillance study that explored the prevalence of intermediate glycopeptide resistance in MRSA bloodstream isolates from our institution, we discovered that vancomycin MICs, assayed by the reference macrodilution (tube) method (9), were 2 μg/ml for a vast majority of our nosocomial isolates. Since these MIC estimates were significantly higher than those currently reported in clinical and epidemiological MRSA surveillance studies, in which the modal vancomycin MIC assayed by the broth microdilution (1, 18, 24, 42, 51) or agar dilution (40, 59) method was 1 μg/ml, we evaluated the impacts of three different susceptibility-testing methods, namely, broth microdilution, tube macrodilution, and a simplified population analysis assay, on glycopeptide MIC distributions for our panel of MRSA isolates. A detailed analysis of parameters that potentially contributed to assay-dependent differences in vancomycin and teicoplanin MIC estimates, such as the inoculum size, time of incubation, and medium composition, was performed. A novel approach, combining broth macrodilution and agar testing, is proposed for discriminating glycopeptide-susceptible from hGISA and GISA isolates.

MATERIALS AND METHODS

MRSA isolates and growth conditions. Two panels of MRSA isolates were used. (i) A panel of borderline glycopeptide-susceptible and glycopeptide-intermediate MRSA isolates ($n = 56$) was selected from a larger collection of MRSA isolates ($n = 225$), which were obtained from bacteremic patients in Geneva University Hospital between January 1995 and December 2003 (1 isolate per septic episode), identified by standard techniques, and stored in skim milk-glycerol at −70°C as described previously (4). This panel of MRSA isolates, which was used for detailed comparative studies of glycopeptide MIC values recorded by three different susceptibility-testing methods, was selected by glycopeptide agar screening of vancomycin- and/or teicoplanin-intermediate resistance, performed as detailed below. (ii) Another panel of 54 clinically significant MRSA isolates was obtained from intraoperative specimens or joint aspirates of 39 patients with orthopedic-device-related infections during the period 2000 to 2008 (16). Glycopeptide-susceptible strains ATCC 29213 and GISA NRS3 (HIP 5827; provided by the Network of Antimicrobial Resistance in *S. aureus* [NARSA] [http://www.narsa.net]) were used as quality control strains. The prototype hGISA strain Mu3 (ATCC 700698) (22, 57) was also provided by NARSA.

Except for glycopeptide agar screening assays (see below), standardized inocula of each MRSA isolate and the quality control strains ATCC 29213, NRS3, and Mu3 were prepared from cultures grown overnight at 37°C in cation-adjusted Mueller-Hinton broth (CAMHB), which were subsequently diluted 1:50 in fresh CAMHB and grown for 3 h at 37°C without shaking. Following adjustment to 0.5 McFarland standard, each log-phase culture was diluted to deliver the final inoculum recommended for each assay, namely, 5×10^5 CFU per ml for the broth microdilution method, 10^6 CFU per ml for tube macrodilution, and 10^6 CFU per agar plate for simplified population analysis, into each antibiotic-containing or control tube or agar plate.

Glycopeptide agar screen. Brain heart infusion (BHI) agar plates containing 2 (BHIV2) or 4 (BHIV4) μg of vancomycin per ml or 5 (BHIT5) or 8 (BHIT8) μg of teicoplanin per ml were seeded in parallel with a 10-μl inoculum of a stationary-phase broth culture, prepared as recommended by the European Antimicrobial Resistance Surveillance System (EAARS) (12), and read after incubation at 37°C for 48 h. Pilot results indicated that 10-μl inocula, delivered by pipette from overnight cultures, yielded more reproducible results than colony suspensions (data not shown). Each agar plate was inoculated with six clinical isolates and two quality control strains, namely, ATCC 29213 and NRS3. Growth of ≥1 colony indicated a positive result. To be validated, each set of results required growth of GISA NRS3 on all four screening media (BHIV2, BHIV4, BHIT5, and BHIT8) and no growth of glycopeptide-susceptible ATCC 29213 on any screening medium.

Broth microdilution MIC. Vancomycin and teicoplanin MICs were tested by broth microdilution according to M07-A8 (9) and M100-S19 (10) guidelines. The final vancomycin and teicoplanin concentrations for MIC determination ranged from 0.06 to 32 μg/ml. All MRSA isolates and the quality control strain *S. aureus* ATCC 29213 were tested at a final inoculum concentration of 5×10^5 CFU/ml, equivalent to 5×10^4 CFU/well (9). The MIC quality control ranges of each daily experiment, recorded with quality control strain ATCC 29213, fell within CLSI-approved ranges for both vancomycin and teicoplanin (10).

Macrodilution MIC. Vancomycin and teicoplanin MICs were determined by tube macrodilution according to M07-A8 (9) and M100-S19 (10) guidelines with slight modifications. Doubling dilutions of glycopeptide concentrations, ranging from 0.5 to 8 μg/ml for vancomycin and 0.5 to 16 μg/ml for teicoplanin, were freshly prepared in CAMHB. Then, each antibiotic-containing or control tube was inoculated with ca. 10^6 CFU of each MRSA isolate or quality control strains ATCC 29213 and NRS3 per ml. The MIC endpoints were read after 24 and 48 h of incubation at 37°C. We used a dual-time-point glycopeptide MIC reading to evaluate the potential presence of slow-growing, "glycopeptide-resistant" subpopulations.

Simplified population analysis. Each MRSA isolate (10^6 CFU), or quality control strains ATCC 29213 and NRS3, was spread in a 200-μl volume on BHI agar plates supplemented with 0.5, 1, 2, 4, or 8 μg of vancomycin or 0.5, 1, 2, 4, 8, or 16 μg/ml of teicoplanin per ml. The plates were incubated at 37°C, and growth was evaluated after 24 and 48 h. Because a single undiluted inoculum was uniformly plated onto BHI agar plates, viable counts were scored in a semiquantitative manner as follows: (i) confluent (≥10^6 CFU); (ii) from >10^3 CFU to semiconfluent (≥10^5 CFU); and (iii) plates with <10^3 CFU, which were quantitatively enumerated. In this simplified population analysis method, the glycopeptide MIC is defined as the lowest antibiotic concentration leading to a ≥99.9% reduction in viable counts (≤10^3 CFU) on BHI agar from the uniformly applied inoculum of 10^6 CFU.

TABLE 1. Comparison of vancomycin and teicoplanin MICs determined by broth microdilution, tube macrodilution, and BHI-agar[a]

Glycopeptide (μg/ml)	No. (%) of isolates with MIC (μg/ml) determined by:									
	Vancomycin					Teicoplanin				
	Microdilution	Macrodilution		Agar (PA)		Microdilution	Macrodilution		Agar (PA)	
		24 h	48 h	24 h	48 h		24 h	48 h	24 h	48 h
0.125	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)
0.25	4 (7)	0 (0)	0 (0)	0 (0)	0 (0)	9 (16)	0 (0)	0 (0)	0 (0)	0 (0)
0.5	14 (25)	0 (0)	0 (0)	0 (0)	0 (0)	13 (23)	1 (2)	0 (0)	0 (0)	0 (0)
1	30 (54)	3 (5)	1 (2)	1 (2)	1 (2)	26 (46)	5 (9)	5 (9)	4 (7)	3 (5)
2	5 (9)	45 (80)	40 (71)	36 (64)	35 (62)	4 (7)	26 (46)	8 (14)	8 (14)	8 (14)
4	3 (5)	6 (11)	13 (23)	17 (30)	18 (32)	3 (5)	22 (39)	36 (64)	28 (50)	23 (41)
8	0 (0)	2 (4)	2 (4)	2 (4)	2 (4)	0 (0)	2 (4)	7 (13)	14 (25)	20 (36)
16	ND	0	0	0	0	ND	0	0	2 (4)	2 (4)

[a] MICs were determined for 56 MRSA blood isolates.

Statistical analysis. Correlations between MIC values generated by two different antibiotic susceptibility methods for each isolate were evaluated by the Spearman rank order correlation coefficient (http://faculty.vassar.edu/lowry/VassarStats.html). MIC differences between two susceptibility test methods (expressed as the ratios of $MIC_{method\ 2}$ to $MIC_{method1}$ for each isolate) were also plotted as a function of $MIC_{method\ 1}$. Correlations between MIC differences and $MIC_{method\ 1}$ for each isolate were evaluated by the Spearman rank order correlation coefficient (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

Preliminary agar screening. A panel of 56 borderline glycopeptide-susceptible and glycopeptide-intermediate isolates was selected by screening 225 MRSA blood isolates on BHI agar supplemented with 2 or 4 μg vancomycin per ml or 5 or 8 μg teicoplanin per ml. Forty-two isolates grew on at least one screening medium, which was BHIT5 for almost all (97%) of them. In contrast, a single isolate was recovered on vancomycin-supplemented, but not teicoplanin-supplemented, agar. Fourteen additional isolates, scored as negative by agar screening but that showed elevated glycopeptide MICs in pilot testing (not shown), were included in the panel of MRSA isolates. A detailed compilation of all glycopeptide-screening results for the 56 MRSA isolates is shown in Table S1 in the supplementary material, which summarizes all glycopeptide MIC data recorded in parallel by microdilution, macrodilution, and simplified population analysis (see below).

Twenty-two of the 41 screen-positive isolates on teicoplanin-supplemented agar grew exclusively on BHIT5 and 19 isolates on both BHIT5 and BHIT8. Fourteen of the teicoplanin-positive isolates also grew on vancomycin-supplemented agar, namely, 8 isolates on BHIV2 only and 6 isolates on both BHIV2 and BHIV4.

Comparison of vancomycin and teicoplanin MICs by broth microdilution and macrodilution. Simultaneous testing of vancomycin and teicoplanin MICs by broth microdilution and tube macrodilution revealed major assay-dependent differences in modal MICs and MIC distributions for each glycopeptide (Table 1). The modal MICs of both vancomycin and teicoplanin for the 56 isolates were 1 μg/ml by microdilution compared to 2 μg/ml by macrodilution. Vancomycin MICs by microdilution were ≤1 μg/ml for 86% compared to 5 and 2% of isolates recorded by macrodilution at 24 and 48 h, respectively (Table 1). While vancomycin MICs by microdilution were 2 and 4 μg/ml for only 9% and 5% of isolates, respectively, they were 2, 4, and 8 μg/ml by macrodilution at 24 h for 80, 11, and 4% of isolates, respectively. At 48 h, vancomycin MICs by macrodilution were 2, 4, and 8 μg/ml for 71, 23, and 4% of isolates, respectively.

Similar assay-dependent differences were observed with teicoplanin MICs that were ≤1 μg/ml by microdilution for 88% compared to 11 and 9% of isolates at 24 and 48 h, respectively, by macrodilution (Table 1). While teicoplanin MICs by microdilution were 2 and 4 μg/ml for 7 and 5% of isolates, respectively, they were 2, 4, and 8 μg/ml at 24 h for 46, 39, and 4% of isolates, respectively, by macrodilution. At 48 h, teicoplanin MICs by macrodilution were 2, 4, and 8 μg/ml for 14, 64, and 13% of isolates, respectively.

Assay-dependent differences in glycopeptide MICs were also regularly recorded with the repeatedly assayed ($n = 12$) glycopeptide-susceptible reference strain ATCC 29213. The modal vancomycin MIC for ATCC 29213 was 2 μg/ml at 24 and 48 h by macrodilution compared to 0.5 μg/ml by microdilution, and the modal teicoplanin MIC for ATCC 29213 was 1 μg/ml at 24 and 48 h by macrodilution compared to 0.25 μg/ml by microdilution.

Additional independently performed experiments (data not shown) confirmed the reproducibility of microdilution-assayed MIC distributions and modal MICs of vancomycin and teicoplanin, shown in Table 1 for the 56 MRSA isolates.

Pairwise comparisons of vancomycin or teicoplanin macrodilution versus microdilution MICs for each isolate indicated that both vancomycin (Fig. 1A) and teicoplanin (Fig. 1C) macrodilution MICs were correlated to some extent with those recorded by microdilution ($P < 0.001$). However, assay-dependent MIC differences for both vancomycin (Fig. 1B) and teicoplanin (Fig. 1D) were not uniform but tended to be greater (ca. 4-fold) for isolates displaying low MICs (≤0.5 μg/ml) by microdilution than for those with higher MICs (ca. 2-fold). For both glycopeptides, MIC differences between the two susceptibility test methods (expressed as the ratios of $MIC_{macrodilution}$ over $MIC_{microdilution}$ for each isolate) were negatively correlated with microdilution MICs ($P < 0.001$). Interestingly, assay-dependent differences were more pronounced for teicoplanin than for vancomycin MICs. Indeed, $MIC_{macrodilution}$ over $MIC_{microdilution}$ ratios of ≥4-fold were recorded with teicoplanin for 68% ($n = 38$) compared to 38% ($n = 21$) of isolates with vancomycin ($P < 0.003$).

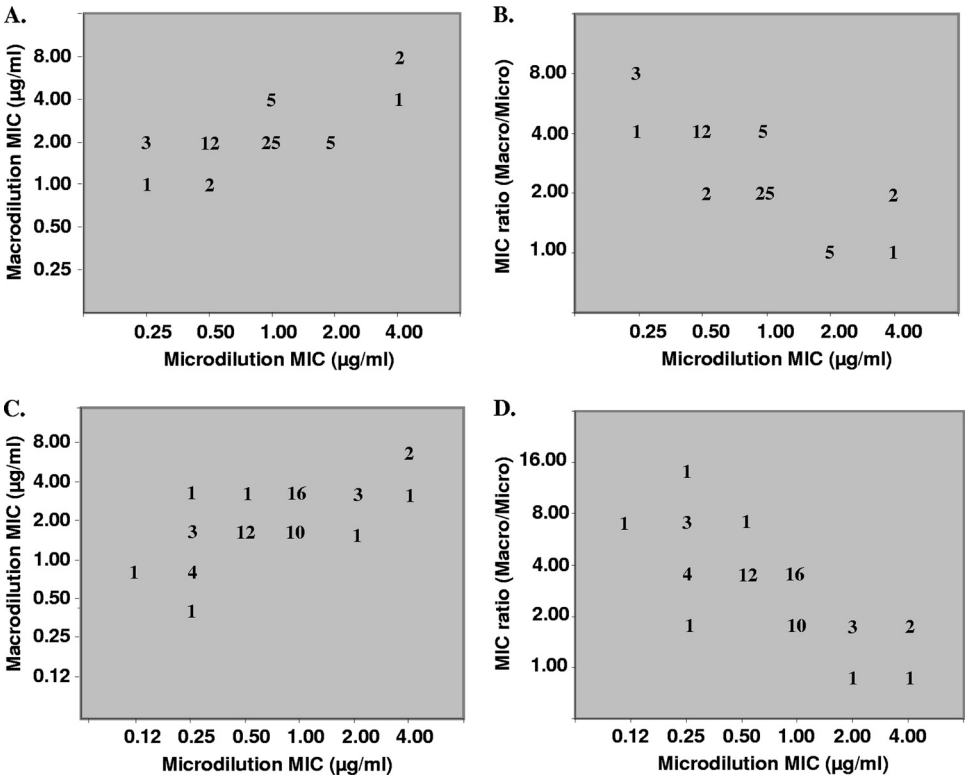

FIG. 1. (A) Pairwise comparison of vancomycin MICs determined at 24 h by macrodilution versus microdilution for each MRSA isolate. (B) Fold increases (expressed as the ratios of $MIC_{macrodilution}$ over $MIC_{microdilution}$ for each isolate) in vancomycin MICs by macrodilution over microdilution are negatively correlated with microdilution MICs ($P < 0.001$). (C) Pairwise comparison of teicoplanin MICs by macrodilution versus microdilution. (D) Fold increases (expressed as the ratios of $MIC_{macrodilution}$ over $MIC_{microdilution}$ for each isolate) in teicoplanin MICs by macrodilution over microdilution are negatively correlated with microdilution MICs ($P < 0.001$).

Combined impact of inoculum size and incubation time on MICs assayed under liquid conditions. While assay-dependent differences in glycopeptide MICs for MRSA isolates were hardly explained by the 2-fold-higher inoculum concentrations used for the macrodilution (10^6 CFU/ml) compared to the microdilution (5×10^5 CFU/ml) assay, these MIC differences more likely resulted from the 20-fold-lower inoculum size (5×10^4 CFU/well) used for the microdilution than for the macrodilution method. To evaluate this parameter, we compared the impacts of two different inoculum sizes, namely, 5×10^4 CFU (low inoculum) and 10^6 CFU (standard inoculum) per ml, on vancomycin MICs by macrodilution. Significant differences in vancomycin MIC distributions were recorded at 24 h for low-inoculum compared to standard-inoculum isolates (Table 2). Furthermore, vancomycin MICs that were 1 μg/ml at 24 h for 55% ($n = 31$) of isolates in the low-inoculum group compared to 12% ($n = 7$) of isolates in the standard-inoculum group increased from 1 to 2 μg per ml at 48 h for 20 of the 31 isolates in the low-inoculum group, as well as for all 7 isolates in the standard-inoculum group with previously recorded vancomycin MICs of 1 μg/ml at 24 h.

The impact of inoculum size on glycopeptide modal MICs was also observed with the repeatedly assayed ($n = 10$) glycopeptide-susceptible reference strain ATCC 29213, for which the modal vancomycin MIC was 1 μg/ml under low-inoculum compared to 2 μg/ml under standard-inoculum conditions. These data demonstrated that both inoculum size and incubation time had a significant combined impact on glycopeptide MICs assayed under liquid conditions.

Pairwise comparisons of vancomycin MICs for each MRSA isolate, assayed by macrodilution under low-inoculum conditions versus microdilution, showed that they were significantly correlated (Fig. 2A). However, assay-dependent differences in vancomycin MICs (expressed as the ratios of $MIC_{macrodilution}$ over $MIC_{microdilution}$ for each isolate) recorded by macrodilution at both 24 (Fig. 2B) and 48 (not shown) h were still negatively correlated ($P < 0.001$) with microdilution MICs. Thus, assay-dependent differences in inoculum size contributed in part, but not entirely, to the vancomycin MIC differ-

TABLE 2. Comparison of vancomycin MICs determined by tube macrodilution on low and standard bacterial inocula[a]

Vancomycin MIC (μg/ml)	No. (%) of isolates with vancomycin MIC (μg/ml) determined in:			
	Low-inoculum group (5×10^4 CFU/ml)		Standard-inoculum group (10^6 CFU/ml)	
	24 h	48 h	24 h	48 h
1	31 (55)	11 (20)	7 (12)	0 (0)
2	20 (36)	39 (70)	43 (77)	50 (89)
4	5 (9)	5 (9)	6 (11)	5 (9)
8	0 (0)	1 (2)	0 (0)	1 (2)

[a] MICs were determined for 56 MRSA blood isolates.

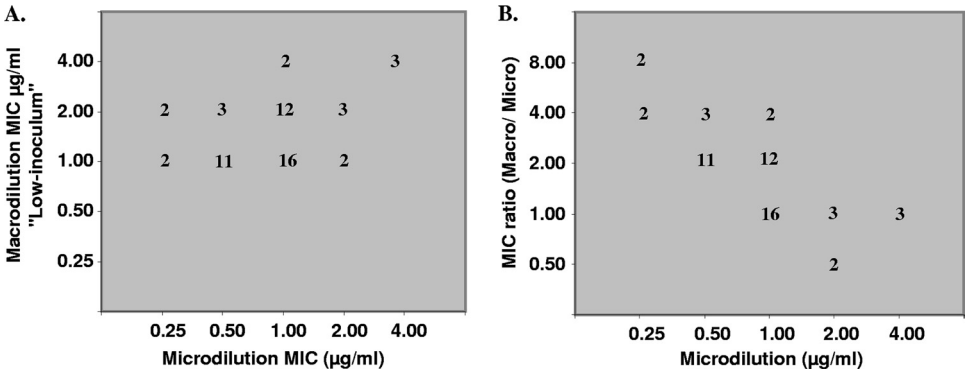

FIG. 2. (A) Pairwise comparison of vancomycin MICs (24 h) by macrodilution assayed under low-inoculum conditions (5×10^4 CFU/ml) versus microdilution for each MRSA isolate. (B) Fold increases (expressed as the ratios of $MIC_{macrodilution}$ over $MIC_{microdilution}$ for each isolate) in vancomycin MICs (24 h) by macrodilution assayed under low-inoculum conditions over microdilution are negatively correlated with microdilution MICs ($P < 0.001$).

ences between microdilution and macrodilution susceptibility-testing methods.

Evaluation of vancomycin and teicoplanin MICs by agar testing. Vancomycin and teicoplanin MICs were also evaluated by a simplified population analysis method on glycopeptide-supplemented BHI agar, performed in parallel with microdilution and macrodilution glycopeptide MIC assays (Table 1). Bacterial growth from a single inoculum of 10^6 CFU, uniformly spread on agar plates supplemented with incremental doubling concentrations of vancomycin, was confluent ($n = 52$) or semiconfluent ($n = 3$) on BHI agar containing 1 μg/ml vancomycin (BHIV1) for all but one MRSA isolate. These data provided evidence that vancomycin MICs were ≥2 μg/ml for 98% of isolates on BHI agar, as observed by macrodilution at 48 h (Table 1). Vancomycin MICs of 2 μg/ml were also consistently recorded for the repeatedly assayed reference strain ATCC 29213, which regularly ($n = 12$) showed confluent growth on BHIV1 agar but only marginal growth (<10 CFU) on BHIV2 agar.

Fifty-one and 2 MRSA isolates showed confluent or semiconfluent growth, respectively, on BHI agar supplemented with 1 μg/ml teicoplanin. Teicoplanin MICs were therefore ≥2 μg/ml for 95% of isolates on BHI agar compared to 91% of isolates by macrodilution at 48 h (Table 1). The teicoplanin MIC range was 1 to 2 μg/ml on BHI agar for the repeatedly assayed ($n = 12$) reference strain ATCC 29213.

Underdetection of GISA by broth microdilution MIC assay. Underestimation of vancomycin and teicoplanin MICs by broth microdilution led to significant underdetection of GISA or hGISA isolates (Table 1 and Fig. 1). While vancomycin MICs were 4 μg/ml by broth microdilution for only three MRSA isolates, which were thus scored as VISA, vancomycin MICs by macrodilution were 4 μg/ml for 6 and 13 isolates at 24 and 48 h, respectively. For 2 of the 3 isolates identified as VISA by microdilution, vancomycin MICs by macrodilution were 8 μg/ml at both 24 and 48 h.

In contrast to broth microdilution, which did not detect any isolate with an elevated teicoplanin MIC (8 to 16 μg/ml), macrodilution scored 2 and 7 isolates at 24 and 48 h, respectively, for which the teicoplanin MIC was 8 μg/ml. While broth microdilution detected three isolates with teicoplanin MICs of 4 μg/ml, which would mark them as teicoplanin resistant according to recently revised EUCAST clinical MIC breakpoints for glycopeptide resistance (13), the macrodilution method detected 22 and 36 isolates for which the teicoplanin MICs were 4 μg/ml at 24 and 48 h, respectively. For a majority ($n = 16$) of the 22 isolates for which the teicoplanin MICs were 4 μg/ml at 24 h by macrodilution, the teicoplanin MICs were only 1 μg/ml by microdilution (Fig. 1C), again emphasizing the major assay-dependent differences in teicoplanin MIC estimates.

A novel approach for discriminating non-GISA from GISA isolates. Since a significant proportion of GISA isolates cannot be detected by standard glycopeptide MIC methods due to their heteroresistance, their identification relies on the presence of resistant subpopulations growing on BHI agar in the presence of ≥2 μg of vancomycin or ≥4 μg of teicoplanin per ml (21, 30). Besides detection on agar by population analysis (21, 24, 30, 51, 57), resistant subpopulations should be detected by their ability to grow within 48 h in a liquid medium supplemented with 2 μg of vancomycin and/or 4 μg of teicoplanin per ml. The sizes and growth rate properties of resistant subpopulations should directly influence vancomycin MIC values for each isolate, which may reach 4 μg/ml as early as 24 h or only at 48 h. In a similar way, teicoplanin-resistant subpopulations should be detected by teicoplanin MICs of ≥8 μg per ml as early as 24 h, or later, at 48 h. The aim of this approach, which does not attempt to differentiate hGISA from GISA isolates, is mainly to discriminate non-GISA from hGISA/GISA isolates.

Accordingly, we propose to detect hGISA/GISA isolates by a macrodilution MIC of 4 μg vancomycin or 8 μg teicoplanin per ml, recorded at 24 or 48 h, and/or confluent or semiconfluent growth within 48 h on BHI agar supplemented with 2 μg of vancomycin and/or 4 μg of teicoplanin per ml; conversely, glycopeptide-susceptible isolates should have vancomycin MICs of ≤2 μg/ml or teicoplanin MICs of ≤4 μg/ml by macrodilution at 48 h and display no growth or only marginal growth (<10^3 CFU) on agar supplemented with 2 μg vancomycin (BHIV2) or 4 μg teicoplanin (BHIT4) per ml. Unfortunately, the recently revised EUCAST clinical breakpoints for teicoplanin MICs (>2 μg/ml), which are linked with the exclu-

sive use of the broth microdilution MIC assay (13), cannot be integrated into our approach.

Evaluation of criteria for assessment of non-VISA isolates. Thirty of the 56 isolates for which vancomycin MICs by macrodilution were still ≤2 μg/ml at 48 h and which showed marginal growth (<10^3 CFU) on BHIV2 agar (see Table S1 in the supplementary material) were scored as non-VISA. Growth was even less than 10^2 CFU on BHIV2 agar for 80% (n = 24) of those isolates.

Further evaluation of criteria for assessing non-VISA isolates was performed by using another panel of clinically relevant isolates that were not preselected by screening on glycopeptide-containing agar. This panel of 54 MRSA, retrieved from patients with orthopedic-device-related infections (16), was composed of a mixture of intraoperative specimens and joint aspirates representing 39 pretherapy and 15 follow-up MRSA isolates. Interestingly, vancomycin MICs by macrodilution were ≤2 μg/ml at 24 and 48 h for all but one isolate (see Table S2 in the supplemental material). The results from these assays were validated by the highly reproducible vancomycin MICs of ≤2 and ≥8 μg/ml for the non-GISA and GISA quality control strains 29213 and NRS3, respectively, recorded by both macrodilution (n = 14) and agar testing (n = 14).

For a single follow-up isolate, the vancomycin MIC by macrodilution increased from 2 to 4 μg per ml from 24 to 48 h and was also 4 μg/ml by agar testing, as indicated by semiconfluent growth on BHIV2 agar. For two other follow-up isolates, vancomycin MICs were 2 μg/ml by macrodilution but 4 μg/ml by agar testing. Notably, all of the other 51 isolates for which the vancomycin MICs were 2 μg/ml by macrodilution showed no growth (n = 24) or marginal growth (<10^3 CFU; n = 27) on BHIV2 agar (see Table S2 in the supplemental material). Thus, there was no significant overlap between the distributions of CFU counts recorded for the 51 non-VISA isolates and those of the 3 isolates that displayed semiconfluent growth on BHIV2 agar, which may be helpful for discriminating non-VISA from potential VISA isolates.

Evaluation of criteria used for detection of GISA isolates. The criteria used to characterize expression of elevated glycopeptide MICs by MRSA isolates were further evaluated by (i) comparing the glycopeptide MICs recorded by macrodilution versus agar testing for each isolate, (ii) comparing the vancomycin and teicoplanin MICs for each isolate, (iii) evaluating the influence of medium composition on the phenotypic expression of elevated vancomycin and teicoplanin MICs by macrodilution, and (iv) evaluating the day-to-day reproducibility of elevated vancomycin (≥4 μg/ml) or teicoplanin (≥8 μg/ml) MICs for each isolate, which is a critical but rarely reported parameter.

Comparison of glycopeptide MICs recorded by macrodilution versus agar testing. In the panel of 56 bacteremic isolates, vancomycin MICs at 48 h were ≥4 μg/ml for 15 isolates by macrodilution and for 20 isolates by agar testing (see Table S1 in the supplemental material). The results from these assays were validated by the highly reproducible vancomycin MICs of 8 μg/ml for the GISA strain NRS3, recorded by both macrodilution (n = 12) and agar testing (n = 12).

Seven and 2 isolates for which the vancomycin MICs were 4 and 8 μg/ml, respectively, by both macrodilution and agar testing were scored as confirmed VISA. For 6 further isolates, elevated vancomycin MICs were detected by macrodilution but not by agar testing. For 11 further isolates, elevated vancomycin MICs were detected by agar testing but not by macrodilution. The significance of these data is discussed below.

Detection of elevated teicoplanin MICs revealed more systematic assay-dependent differences. Macrodilution and agar testing identified 7 and 22 isolates, respectively, for which the teicoplanin MICs were ≥8 μg/ml at 48 h (see Table S1 in the supplemental material). Six of the 7 isolates for which the teicoplanin MICs were 8 μg/ml by macrodilution also displayed elevated teicoplanin MICs (≥8 μg/ml) by agar testing. The ca. 3-fold-higher number of isolates for which teicoplanin MICs were 8 μg/ml by agar testing compared to macrodilution confirmed the markedly increased sensitivity of the agar over liquid MIC method for detecting isolates with reduced teicoplanin susceptibility. We also noticed that for 16 isolates, the teicoplanin MICs (8 μg/ml) at 48 h by agar testing were systematically higher than those (4 μg/ml) assayed in parallel by macrodilution (see Table S1 in the supplemental material).

Teicoplanin MICs (8 to 16 μg/ml) for the GISA strain NRS3 were highly reproducible (n = 12) by both macrodilution and agar testing.

Pairwise comparison of elevated vancomycin and teicoplanin MICs for each isolate. Isolates with elevated teicoplanin MICs (8 μg/ml) were not uniformly cross-resistant to vancomycin. While a first group of 8 isolates (no. 49 to 56) for which teicoplanin MICs were 8 μg/ml showed cross-resistance to vancomycin (vancomycin MICs ≥ 4 μg/ml), a second group of 10 isolates (no. 21 to 30) with teicoplanin MICs of 8 μg/ml by agar testing still displayed vancomycin-susceptible MICs of 2 μg/ml by both agar testing and macrodilution (see Table S1 in the supplemental material).

Influence of liquid medium composition on elevated vancomycin and teicoplanin MICs by macrodilution. To evaluate whether the increased detection of GISA isolates by the agar over the macrodilution MIC method was due to the different chemical compositions of BHI and CAMHB, we assayed in parallel vancomycin and teicoplanin MICs in BHI broth and CAMHB. Overall, BHI slightly promoted expression of elevated vancomycin MICs (≥4 μg/ml) at 48 h for 25 compared to 17 isolates in CAMHB (see Table S3 in the supplemental material).

In contrast to vancomycin, BHI did not significantly influence detection of isolates with elevated teicoplanin MICs compared to CAMHB (see Table S3 in the supplemental material).

Finally, glycopeptide MICs that were recorded for the prototype hGISA strain, Mu3 (21, 22, 57), by macrodilution, assayed in parallel in BHI broth and CAMHB, were highly reproducible (n = 4) for both vancomycin (4 μg/ml) and teicoplanin (8 μg/ml) and were identical in CAMHB and BHI broth (see Table S3 in the supplemental material).

Day-to-day reproducibility of elevated vancomycin or teicoplanin MICs for each isolate. The day-to day reproducibility of susceptible or elevated vancomycin (≥4 μg/ml) and teicoplanin (≥8 μg/ml) MICs for the 56 MRSA isolates, assayed in three independent experiments, is shown in Table S4 in the supplemental material. While experiments A (see Table S1 in the supplemental material) and B represent two separately performed rounds of glycopeptide MICs for the same isolates by macrodilution and simplified population analysis, the results

from experiment C refer to the comparative glycopeptide MICs recorded in parallel by macrodilution in CAMHB and BHI broth (see Table S3 in the supplemental material).

Six MRSA isolates displayed reproducible vancomycin MICs of 4 to 8 μg/ml by both macrodilution and agar testing in all individual assays ($n = 6$). Furthermore, elevated vancomycin MICs of ≥4 μg/ml were recorded in 2 to 5 assays for 23 further isolates but only once for 11 other isolates.

Significant day-to-day variability of vancomycin MICs was also recorded for 12 of the 30 isolates initially scored as non-VISA in experiment A. Interestingly, a common property of the 12 isolates that failed to confirm the initially determined non-VISA phenotype in experiment A was elevated teicoplanin MICs (8 μg/ml) by agar testing in experiment A and/or experiment B. On the other hand, vancomycin MICs of 2 μg per ml were consistently recorded for 18 isolates in all assays.

The day-to-day reproducibility of elevated teicoplanin MICs was superior to that of vancomycin MICs (see Table S4 in the supplemental material). In particular, teicoplanin MICs of 8 μg per ml by agar testing were recorded for 14 isolates in both experiments A and B. As in experiment A, a higher number of isolates ($n = 23$) with teicoplanin MICs of 8 μg/ml were detected in experiment B by agar testing than by macrodilution ($n = 11$). A systematic trend toward 2-fold-higher teicoplanin MICs (8 μg/ml) by agar testing, compared to those (4 μg/ml) recorded by macrodilution, was also found for 13 isolates in experiment B. Nevertheless, teicoplanin MICs of ≥4 μg/ml were consistently recorded for 32 isolates by macrodilution and agar testing in all assays.

Highly reproducible vancomycin and teicoplanin MIC estimates were recorded with the quality control strains 29213 and NRS3. The vancomycin and teicoplanin MIC ranges were invariably 1 to 2 μg/ml for the non-GISA strain 29213 and 8 to 16 μg/ml for the GISA strain NRS3 (data not shown).

Finally, the day-to-day reproducibility of glycopeptide MICs was also evaluated on independently thawed samples of hGISA strain Mu3 (see Table S5 in the supplemental material). Interestingly, vancomycin MICs assayed by macrodilution for strain Mu3 showed significant day-to-day variability from 2 to 4 μg per ml, while MIC estimates by agar testing were more regularly scored as 4 μg per ml. In contrast, the day-to-day reproducibility of teicoplanin MICs (≥8 μg per ml) assayed in parallel on the same independently thawed specimens was excellent and consistent with previous reports (21, 22). Altogether, the day-to-day variability of vancomycin MICs assayed on independently thawed specimens of strain Mu3 contrasted with the highly reproducible MIC estimates recorded in experiment C (see Table S3 in the supplemental material), which were also assayed on different days but from liquid subcultures derived from a single agar-plated, thawed specimen.

DISCUSSION

Simple, reliable assays for the phenotypic detection of hGISA/GISA isolates are urgently needed for therapeutic decisions. The distinction of GISA versus non-GISA isolates is challenging considering the lack of accuracy intrinsic in antibiotic MIC determinations, whose reproducibility may vary by plus or minus one 2-fold dilution (49, 53), in particular for isolates with the most highly heterogeneous expression of glycopeptide-intermediate resistance (24).

While the recent reduction of vancomycin (10) and teicoplanin (13) MIC breakpoints for *S. aureus* based on glycopeptide susceptibility surveys was appropriate (51, 59), key information is still lacking on the sensitivity and accuracy of different susceptibility testing methods for evaluating glycopeptide MICs that are critical for detection of hGISA/GISA (6, 40, 51, 59). Moreover, the potential impact of the lowered glycopeptide MIC breakpoints on the currently established distinction between hGISA and GISA has not been addressed.

Despite indications that the broth microdilution assay uses inadequate inocula (51) and presumably too short incubation periods for detecting hGISA, this method is still recommended by the CDC (6) and EUCAST (13) as a reference method for estimating glycopeptide MICs (49). While alternative GISA detection methods, such as modified Etest methods and population analysis profiles, use more appropriate bacterial inocula and incubation periods, they generate complex results that can hardly be integrated into the current schemes of glycopeptide MIC breakpoints.

To develop more sensitive detection methods for hGISA/GISA, we slightly modified the standard macrodilution MIC assay method, which uses higher inocula than broth microdilution, by introducing sequential MIC readings at 24 and 48 h, which aimed to improve the detection of slowly growing resistant subpopulations. The systematic differences in both vancomycin and teicoplanin MICs recorded by the macrodilution versus the microdilution method were more pronounced than anticipated, in particular for isolates with low glycopeptide MICs. Not only the smaller inoculum size used for the microdilution than for the macrodilution method, but other, still undefined parameters contributed to assay-dependent differences in glycopeptide MICs, as shown by vancomycin MIC distributions assayed by macrodilution under low-inoculum conditions compared to microdilution for each isolate.

The reliability of glycopeptide MICs evaluated by macrodilution for detecting hGISA/GISA phenotypes in MRSA isolates was supported by a simplified population analysis method, using an inoculum equivalent to that used for macrodilution MICs for each isolate and a standard scale of incremental doubling glycopeptide concentrations. The occurrence of confluent or semiconfluent growth for ≥95% of isolates on agar supplemented with 1 μg vancomycin or teicoplanin per ml indicated that their glycopeptide MICs were 2 μg/ml, as recorded by macrodilution.

Altogether, our results may potentially explain the occurrence of higher rates of vancomycin treatment failures for bacteremic patients infected with MRSA isolates for which vancomycin MICs (2 μg/ml) were still in the susceptible range compared to those with lower vancomycin MICs (<2 μg/ml) (3, 11, 18–20, 31, 32, 34, 35, 38, 43, 46, 51). Retrospectively, we can speculate that a substantial proportion of isolates for which vancomycin MICs were scored as 2 μg per ml by microdilution (32, 35, 36, 43) were undetected VISA or hVISA, which could potentially contribute to the higher rates of vancomycin failure (1).

The increased sensitivities of the macrodilution and agar testing methods over the microdilution assay were combined to develop a novel approach for discriminating non-GISA from

TABLE 3. Proposed criteria for differentiating hVISA/VISA from non-VISA isolates

Category	Vancomycin MIC (μg/ml) (macrodilution)	And/or	Agar testing	
			Growth on BHIV2[a]	Growth on BHIT4[b]
Non-VISA	≤2	And	$<10^3$ CFU	$<10^3$ CFU
Potential VISA	≥4	Or	SC2[c] or C2[d]	SC4 or C4
Proven VISA	≥4	And	SC2 or C2	SC4 or C4

[a] Number of CFU/plate from 200-μl inocula (10^6 CFU/plate) on BHI agar supplemented with 2 μg of vancomycin (BHIV2) per ml.
[b] Number of CFU/plate from 200-μl inocula (10^6 CFU/plate) on BHI agar supplemented with 4 μg of teicoplanin (BHIT4) per ml.
[c] Semiconfluent growth on BHIV2 (SC2) or BHIT4 (SC4).
[d] Confluent growth on BHIV2 (C2) or BHIT4 (C4).

GISA/hGISA isolates. Detailed analysis of parameters influencing the GISA phenotype provided evidence for a more irregular expression of vancomycin than teicoplanin-intermediate resistance, assayed by macrodilution and agar MIC-testing methods. The variable expression of vancomycin MICs used for detection of hVISA/VISA phenotypes, namely, a 2-fold change from 2 to 4 μg of vancomycin per ml, was essentially observed during interday comparisons of subcultures from independently thawed specimens of some clinical isolates. A similar finding was obtained with the prototype hVISA strain Mu3. In contrast, the reproducibility of vancomycin MIC estimates was quite satisfactory for same-day assays or for different-day assays using inocula derived from a single primary culture plate of a thawed specimen, such as was observed with the hGISA strain Mu3. Further studies are required to elucidate the mechanisms contributing to the variable VISA phenotype of independently thawed specimens.

Although the agar testing method showed a markedly increased sensitivity for detection of teicoplanin-intermediate isolates compared to macrodilution, this difference was not explained by the presence of BHI in the agar medium but seemed to involve other, still unknown parameters. While assaying teicoplanin MICs may represent a useful first step toward detection of glycopeptide-intermediate resistance, it is by no means sufficient to predict a VISA or hVISA phenotype for each clinical isolate, as indicated by our results and previous reports (6, 21, 30). While clinical isolates displaying confirmed VISA phenotypes are almost uniformly cross-resistant to teicoplanin, cross-resistance from teicoplanin to vancomycin is by no means universal (41). In our study, a significant proportion of clinical isolates showing teicoplanin-intermediate resistance by agar testing were scored as susceptible to vancomycin. The molecular mechanisms responsible for differences in cross-resistance need to be explored by detailed studies of isolates resistant to teicoplanin alone compared to those resistant to both glycopeptides, which may help to identify potentially useful molecular markers (41).

In conclusion, a summary of major phenotypic criteria allowing the discrimination of hVISA/VISA from non-VISA isolates is presented in Table 3. (i) Non-VISA isolates are characterized by vancomycin MICs of ≤2 μg per ml by macrodilution and marginal growth on BHIV2 agar and do not display significantly elevated teicoplanin MICs. (ii) Possible hVISA/VISA isolates are identified by vancomycin MICs of ≥4 μg per ml by macrodilution but not agar testing, or vice-versa, and by displaying elevated teicoplanin MICs (≥8 μg/ml). Assessment of their hVISA/VISA or non-VISA phenotype can be achieved only by repeat testing of the isolates. (iii) Confirmed hVISA/VISA isolates are identified by elevated vancomycin MICs of ≥4 μg per ml by both macrodilution and agar testing, combined with elevated teicoplanin MICs (≥8 μg/ml). A strict distinction between hVISA and VISA is not justified for those isolates that may switch from hVISA to VISA phenotypes or vice versa in different-day experiments.

Further studies are required to characterize in more detail the comparative merits of different glycopeptide MIC-testing and VISA/hVISA detection methods. Comparative studies should also be planned for a more detailed revision of teicoplanin MIC breakpoints, in line with the most sensitive glycopeptide susceptibility-testing methods for *S. aureus*.

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